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TECHNICAL MEMORANDUM 24

IMMUNOLOGICAL STUDIES WITH BACILLUS ANTHRACIS

- I. Bacillus anthracis Aerosol Challenge
of Guinea Pigs

Vaccinated with Protective Antigen

SEPTEMBER 1962

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U.S. ARMY CHEMICAL-BIOLOGICAL-RADIOLOGICAL AGENCY
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Fort Detrick, Maryland

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ABSTRACT

This study indicated that three separate injections of Bacillus anthracis protective antigen provided a higher level of immunity than one injection when guinea pigs were challenged by the respiratory route with two different strains (V1B and NH₆) of Bacillus anthracis. Based on LD₅₀ values obtained with control animals, protective antigen provided a higher level of resistance against intracutaneous route of challenge than the respiratory. Immunized animals challenged intracutaneously with strain NH₆ displayed a much lower level of resistance against this route than that observed for strain V1B. Averaged LD₅₀ values obtained with control guinea pigs indicated that strain V1B was more virulent via the respiratory route than strain NH₆. In contrast, strain NH₆ was slightly more virulent than strain V1B for control animals via the intracutaneous route.

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I. INTRODUCTION

Little information can be found in the open literature concerning the respiratory challenge with Bacillus anthracis of guinea pigs previously immunized via the subcutaneous route with B. anthracis protective antigen. Russian workers¹ have used protective antigen in the vaccination of sheep and found it to be on a par with live vaccine when these animals were challenged subcutaneously. Recent studies by F. Klein et al² using B. anthracis protective antigen, have shown it to be highly protective for guinea pigs when used in conjunction with live vaccines. In Klein's studies, however, all animals were immunized and challenged via the intraperitoneal route.

The purpose of the present study was to determine the ability of an aluminum-hydroxide-gel-adsorbed B. anthracis protective antigen (derived from anaerobic culture) to protect guinea pigs (immunized subcutaneously) against large aerosol doses of virulent B. anthracis spores.

II. MATERIALS AND METHODS

A. IMMUNIZATION AND ANTIBODY TITER PROCEDURES

The vaccination schedule for this study was divided into two phases. In Phase I, guinea pigs were inoculated subcutaneously with 0.5 milliliter of vaccine containing approximately 10 milligrams of crude antigen. This vaccine was obtained from Dr. Milton Puziss, Medical Investigation Division. The description of the method used to develop this vaccine has been recently submitted for publication.³ In Phase II, guinea pigs were inoculated three times (0.5 milliliter per injection) at two-week intervals over a period of four weeks.

Blood sera from all animals were tested for anthrax antibody titers three days prior to challenge. The serological test used to measure antibody titer is referred to as the "agar gel precipitation test" and is a modification of a test⁴ described by C. B. Thorne and F. C. Belton in 1957.⁵ All serological tests were conducted by Mr. John G. Ray, Jr., Medical Investigation Division.

B. CHALLENGE PROCEDURES

Two strains of B. anthracis spore cultures were used in the challenging of immunized and control animals. These strains were designated as V1B and NH6. Both strains were highly virulent for guinea pigs and monkeys by the respiratory or intracutaneous route.

In Phase I, guinea pigs were challenged via the respiratory route at two, three, and four weeks post-vaccination. In Phase II, groups of animals were challenged separately by both the respiratory and intracutaneous route, two weeks after the third injection of antigen or six weeks after the first injection.

Respiratory challenge of guinea pigs was carried out in the Reyniers chamber, by exposing them to aerosols of B. anthracis spores generated by the UCTL atomizer (particle number median diameter, 2.5 microns) at a temperature of $25^{\circ} \pm 2^{\circ}\text{C}$ and relative humidities of 28 ± 3 per cent.

Calculated inhaled doses for guinea pigs in both experimental phases ranged from 2×10^3 to 4×10^5 spores. From 6 to 10 animals were exposed at a point, and aerosol dose was controlled by varying animal exposure time. All aerosols were sampled with Shipe impingers containing 25 milliliters of sterile distilled water as collecting fluid. Concentration of spores per liter of aerosol was calculated from counts of colonies grown on a nutrient agar plate that had been streaked previously with impinger fluid and incubated twenty-four hours at 37°C .

III. RESULTS AND DISCUSSION

Antibody titers, respiratory LD₅₀ values, and 95 per cent confidence limits obtained from immunized and control animals tested in Phase I of this study are recorded in Table I. It can be seen from these results that guinea pigs challenged (with either strain of anthrax) two weeks after vaccination gave respiratory LD₅₀ values that were approximately 2½ logs greater than that obtained with control animals, indicating that the B. anthracis antigen at this point offered some protection to guinea pigs against respiratory challenge. On the third and fourth week post-vaccination, animals challenged with strain V1B showed LD₅₀ values that did not differ significantly from values obtained with control animals. Respiratory LD₅₀ values obtained at three and four weeks with strain NH₆, however, showed no significant change from the two-week values, as seen in the case of strain V1B. This finding indicated that the B. anthracis antigen was able to produce a higher level of protection over a longer period of time against strain NH₆ than it did for strain V1B, when only one injection of antigen was administered.

It is interesting to note that decrease in protection against strain V1B occurred simultaneously with a gradual drop in antibody titer over the four-week period. An observation of definite significance was the fact that strain V1B was more virulent via the respiratory route for guinea pigs than strain NH₆, as demonstrated by the LD₅₀ values.

Respiratory LD₅₀ values obtained in Phase II are shown in Table II. These results indicated, compared with those in Table I, that with either strain of anthrax, three inoculations of antigen afforded more protection to the guinea pig against B. anthracis spores than one inoculation. This observation was borne out by the fact that the respiratory LD₅₀ values for both strains obtained two weeks after the third vaccination (Table II) were approximately a log greater than those obtained two weeks after one vaccination (Table I).

LD₅₀ values obtained with immunized animals challenged intracutaneously were extremely interesting. In the case of strain V1B, the immunity afforded by protective antigen was much greater than that observed with animals challenged by the respiratory route. This statement is borne out by the fact that the intracutaneous LD₅₀ for immunized animals obtained with strain V1B (Table II) was approximately five logs greater than the intracutaneous LD₅₀ values obtained for control animals. Conversely, the intracutaneous LD₅₀ value obtained with strain NH₆ for immunized animals was only three logs greater than the LD₅₀ value obtained for control animals. This difference in protection observed for the two strains via the intracutaneous route can be logically explained on the basis that strain NH₆ was shown to be more virulent for control guinea pigs via the

TABLE I. RESULTS OF RESPIRATORY CHALLENGE OF GUINEA PIGS VACCINATED
ONE TIME WITH B. ANTHRACIS PROTECTIVE ANTIGEN

Time Elapsing Between Vaccination and Challenge, weeks	Vaccinates			Controls ^a	
	Average Antibody Titer	Respiratory LD50, spores	95% Confidence Limits, spores	Respiratory LD50, spores	95% Confidence Limits, spores
Challenge Strain VLB					
2	1:32	2.3 x 10 ⁵	8.6 x 10 ⁴ to 9.0 x 10 ⁵	6.5 x 10 ³	3.9 x 10 ³ to 1.3 x 10 ⁴
3	1:16	5.2 x 10 ⁴	5.5 x 10 ³ to 1.2 x 10 ⁶	5.8 x 10 ⁴	3.6 x 10 ⁴ to 2.1 x 10 ⁵
4	1:8	3.9 x 10 ⁴	3.4 x 10 ³ to 5.4 x 10 ⁴	9.8 x 10 ³	5.5 x 10 ³ to 2.1 x 10 ⁴
Challenge Strain NH6					
2	1:32	3.8 x 10 ⁵	2.1 x 10 ⁵ to 7.1 x 10 ⁵	4.4 x 10 ⁴	1.3 x 10 ⁴ to 1.5 x 10 ⁵
3	1:16	1.0 x 10 ⁵	2.7 x 10 ⁴ to 6.6 x 10 ⁵	5.0 x 10 ⁴	3.1 x 10 ⁴ to 8.1 x 10 ⁴
4	1:±16	2.2 x 10 ⁵	1.2 x 10 ⁵ to 4.0 x 10 ⁵	7.0 x 10 ⁴	3.9 x 10 ⁴ to 1.2 x 10 ⁵

a. Controls prior to aerosol exposure had negative antibody titers, as measured by the agar gel precipitation test.

TABLE II. RESULTS OF RESPIRATORY AND INTRACUTANEOUS CHALLENGE OF GUINEA PIGS
VACCINATED THREE TIMES WITH B. ANTHRACIS PROTECTIVE ANTIGEN

Challenge Route	Vaccinates			Controls ^a /	
	Average Antibody Titer	LD50 Values, spores	95% Confidence Limits, spores	LD50 Values, spores	95% Confidence Limits, spores
Respiratory	1:64	Challenge Strain VLB			
		1.1 x 10 ³	4.3 x 10 ⁵ to 5.2 x 10 ³	3.0 x 10 ³	2.2 x 10 ³ to 4.2 x 10 ⁴
Intracutaneous	1:64	1.7 x 10 ³	8.7 x 10 ³ to 4.1 x 10 ³	12.0	9 to 17
		Challenge Strain NH6			
Respiratory	1:64	1.8 x 10 ³	3.9 x 10 ⁵ to 3.6 x 10 ³	3.8 x 10 ⁴	1.7 x 10 ⁴ to 6.8 x 10 ⁴
Intracutaneous	1:64	3.6 x 10 ³	2.3 x 10 ² to 5.7 x 10 ³	3.0	2.0 to 7.0

a. Controls prior to challenge had negative antibody titers, as measured by the agar gel precipitation test.

intracutaneous route than strain V1B. A final observation that was not anticipated was the finding that the "Time-to-Death" period for immunized animals was not significantly different from that observed for control animals.

Any attempt to compare or extrapolate these data with immunogenic results obtained with humans must take into consideration the fact that the amount of antigen administered to animals in this study was on a weight basis many-fold larger than that administered to personnel working with B. anthracis at Fort Detrick.

Preliminary B. anthracis immunity studies with strain V1B are now being conducted with rhesus monkeys. Results from these studies to date suggest that the B. anthracis protective antigen affords a much greater protection for rhesus monkeys challenged by the respiratory route than that demonstrated with guinea pigs.

IV. SUMMARY

In this study, guinea pigs were immunized with Bacillus anthracis protective antigen adsorbed on aluminum hydroxide. Animals were vaccinated subcutaneously with varying amounts of antigen and then challenged separately via the respiratory and intracutaneous routes with spores of two virulent strains (V1B and NH6) of B. anthracis. The results of this study demonstrated the following trends:

(a) Guinea pigs immunized with one subcutaneous inoculation of protective antigen and then challenged by the respiratory route with strain V1B demonstrated a definite drop in resistance to B. anthracis over a four-week post-vaccinal period, as indicated by a drop in the respiratory LD50 value from 2.3×10^5 to 3.9×10^4 spores. Immunized animals challenged comparably with strain NH6 did not show a drop in the level of resistance over the same time period.

(b) LD50 values obtained with control animals indicated that strain V1B was more virulent for guinea pigs via the respiratory route than strain NH6.

(c) Guinea pigs vaccinated subcutaneously three times over a four-week period displayed a higher level of resistance to respiratory challenge with both strains of B. anthracis than that obtained with one vaccination. This observation was confirmed by the fact that the respiratory LD50 value for immunized animals was approximately one log greater than that obtained for one vaccination.

(d) LD50 values obtained with immunized animals challenged intracutaneously with strain V1B indicated that a higher level of immunity was obtained against this route of challenge than observed via the respiratory route, on the basis of LD50 values observed with control guinea pigs.

(e) LD50 values obtained with immunized animals challenged intracutaneously with strain NH6 indicated that a much lower level of immunity was obtained with strain NH6 than with strain V1B against this route of challenge.

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